Method for the Production and Characterization of Tomato Cutin Oligomers

A series of oligomeric fragments can be obtained by partial alkaline hydrolysis of cutin. The oligomers obtained from tomato cutin were analyzed by high-performance liquid chromatography–mass spectrometry and the major products were characterized as linear dimeric esters of ω,8-, ω,9-, and ω,10-dihydroxyhexadecanoic acids, the major monomers of tomato cutin. Analysis of the products obtained from tomato cutin by treatment with cutinase suggests an exo mode of depolymerization for this enzyme.

Keywords: *Cutin; oligomers; tomato; dihydroxyhexadecanoate; depolymerization*

INTRODUCTION

Cutin is a complex, hydrophobic polymer that is a major constituent of the plant cuticle. This polymer and the cuticular waxes provide a protective barrier between the plant and its environment. It has been hypothesized that the cuticle protects the plant from desiccation and may act as a barrier to microbial and insect pest invasion (Kolattukudy, 1980). There have been numerous studies in which the monomers of various plant cutins have been characterized (Holloway, 1982). These studies show a wide variation in monomer composition among different plant species; however, the predominant monomers are generally substituted C_{16} or C_{18} ω-hydroxy fatty acids. Sites of cross-linking through hydroxyl substituents present in the interior of the fatty acid chain (usually at C9 or C10) have been inferred from the products of hydrolysis of cutins that have been chemically modified prior to hydrolysis (Deas and Holloway, 1977; Kolattukudy, 1977). Although a model for the cutin polymer based on monomer composition has been proposed (Kolattukudy, 1977), only recently has there been direct experimental data for the intact polymer. These studies include solid state NMR of purified lime cutin (Zlotnik-Mazori and Stark, 1988) and potato suberin (Garbow et al., 1989) and Fourier transformed IR examination of the intact cuticle (Chamel and Marechal, 1992; Ramirez et al., 1992).

A more detailed examination of the polymer structure has been initiated in this laboratory as part of an investigation of the breakdown of cutin by bacterial cutinases (Fett et al., 1992a,b). Recently an HPLC method has been described by Gerard et al. (1992a) in which underivatized cutin monomers are separated on the basis of increasing polarity. An HPLC–MS method based on this HPLC protocol has now been developed in our laboratory to characterize cutin oligomers obtained by partial alkaline hydrolysis of cutin. A description of the analytical procedure and its application to the characterization of the structure of tomato cutin is presented herein.

EXPERIMENTAL PROCEDURES

General Procedures. Tomato cutin was prepared as previously described (Gerard et al., 1992b). HPLC–MS was carried out on a Model 5989A mass spectrometer interfaced to a Model 1050 HPLC through a Model 59980B particle-beam separator (all from Hewlett-Packard). GC–MS data were obtained on the same spectrometer interfaced with a Hewlett-Packard 5890 GC fitted with a Hewlett-Packard 12 m × 0.33 μm HP1 (methyl silicone) column. The column temperature was programmed from 125 to 250 °C at 4 °C min^{-1}. The injector (splitless) and the GC–MS interface temperatures were 250 °C.

A Varian 200 MHz spectrometer, operated at 50 MHz, was used to obtain ^{13}C NMR and attached proton test (APT) NMR. Samples were dissolved in $CDCl_3$ with TMS as the internal reference. Thin-layer chromatography was carried out on 20 cm × 20 cm silica gel plates containing fluorescent indicator (Analtech). Column chromatography was run on a 3 cm × 35 cm silica (60 A) (Woelm) column. Size exclusion HPLC was carried out on a TSK G2000HXL column (Supelco) interfaced with a Varex evaporative light scattering ELSD II detector. The column was eluted with tetrahydrofuran (THF) at a flow rate of 0.5 mL min^{-1}. All solvents were of high purity grade from Burdick and Jackson.

Base Hydrolysis of Tomato Cutin. Tomato cutin (30 mg) was treated at ambient temperature with 1 mL of 1.5 M methanolic KOH diluted with 0.2 mL of H_2O. Aliquots (100 μL) were removed at 0.25, 0.5, 1.0, 2.0, and 4.0 h. The aliquots were neutralized with glacial acetic acid and then diluted to 1 mL with H_2O and extracted with 1 mL of $CHCl_3$ (two times). The combined $CHCl_3$ extracts were washed with H_2O (1 mL) and concentrated to dryness under a stream of nitrogen, and the residue was dissolved in 200 μL of $CHCl_3$/MeOH (95:5).

Enzymatic Hydrolysis of Tomato Cutin. Tomato cutin (30 mg) was treated with 100 μg of partially purified cutinase enzyme isolated from *Fusarium solani* under conditions previously described (Gerard et al., 1992b). Aliquots were removed at 0.5, 1.0, 2.0, and 24 h. The products were isolated as described above except for the addition of acetic acid. Analyses were carried out on the free acids and the corresponding methyl esters prepared by reaction with ethereal diazomethane.

HPLC–MS Analysis. HPLC conditions for the analysis of cutin monomers (Gerard et al., 1992b) were used for the HPLC–MS analysis. The particle-beam chamber was maintained at 65 °C. The mass spectrometer was run in the chemical ionization mode with NH_3 as the ionization gas. The ionization chamber temperature was 250 °C.

Isolation of Dihydroxyhexadecanoic Acid Dimer. Tomato cutin (30 mg) was repeatedly treated with 1.5 M methanolic KOH as described above. After each treatment, the cutin solution was filtered and the precipitate washed with MeOH. The $CHCl_3$ extracts from each treatment were combined and subjected to column chromatography. The column was eluted with 250 mL of hexane/2-propanol/acetic acid (90:

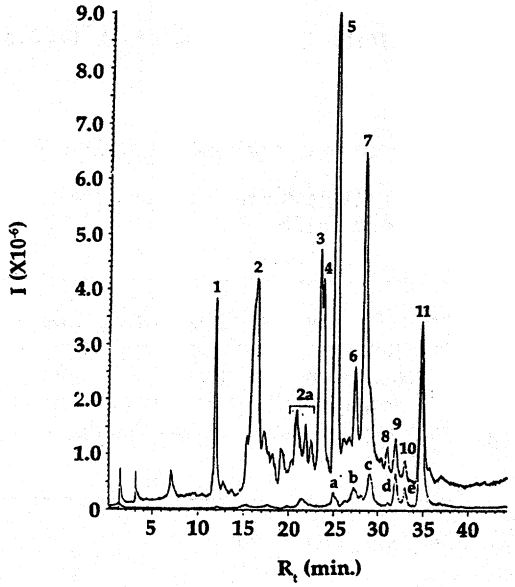

Figure 1. HPLC–MS of 0.5 h, 1.5 M aqueous methanolic KOH hydrolysis of tomato cutin. Peaks 1–11 are in the M_r range 100–1500; peaks a–f are in the M_r range 500–1500.

Table 1. Composition of Partially Hydrolyzed Tomato Cutins Determined by HPLC–MS

peak[a]	RR_t	M_r	[B][b]	compound	%
1	1.00	286	304	dihydroxyhexadecane	6.0
2	1.36	300	318	ω-hydroxyoctadecanoic acid	1.0
3	1.91	272	290	ω-hydroxyhexadecanoic acid	0.9
4	1.94	286	304	n[c],oxo-ω-hydroxyhexadecanoic acid	0.9
5	2.09	330	330	methyl n,ω-dihydroxyoctadecanoate	2.1
6	2.23	302	302	methyl n,ω-dihydroxyhexadecanoate	0.9
7	2.33	302	302	methyl n,ω-dihydroxyhexadecanoate	0.9
8	2.53	302	302	methyl n,ω-dihydroxyhexadecanoate	25.1
9	2.61	288	302	n,ω-dihydroxyhexadecanoic acid	11.8
10	2.73	288	306	n,ω-dihydroxyhexadecanoic acid	33.8
11(f)	2.85	572	573	dimer	7.5

[a] See Figure 1. [b] Base peak of the CI_{NH3}-MS spectrum. [c] The hydroxyl group is assumed to be ω for the monohydroxy compounds as well as for one of the hydroxyls in the dihydroxy compounds with the other hydroxyl (n) at C8, C9, or C10 on the basis of previous results (Gerard et al., 1992b).

Table 2. Monomer (m/z 100–1500) and Dimer (m/z 500–1500) Total Ion Current (TIC) vs Sampling Time

	sampling time			
	0.5 h	1 h	2 h	4 h
m/z 100–1500 ($\times 10^{-10}$) TIC[a]	1.4	1.7	2.5	4.5
m/z 500–1500 ($\times 10^{-9}$) TIC	1.8	1.6	0.2	0.1

[a] Total ion current.

10:0.2) followed by 500 mL of hexane/2-propanol/acetic acid (84:16:0.2); 10 mL fractions were collected. The fractions were monitored by HPLC–MS. Fractions rich in the dimer were further purified by preparative HPLC using the last mentioned solvent system.

Dimer Derivatization and Hydrolysis. The purified dimer was acetylated with acetic anhydride/pyridine (1:1, 70 °C for 3 h). Hydrolysis of the dimer with 1.5 M aqueous methanolic KOH for 24 h at room temperature yielded a product which was converted to the methyl ester with ethereal diazomethane and then silylated with bis(trimethylsilyl)-acetamide (Alltech).

RESULTS AND DISCUSSION

Tomato cutin was chosen to study the feasibility of using partial chemical hydrolysis or enzymatic hydrolysis in combination with MS to probe the structure of

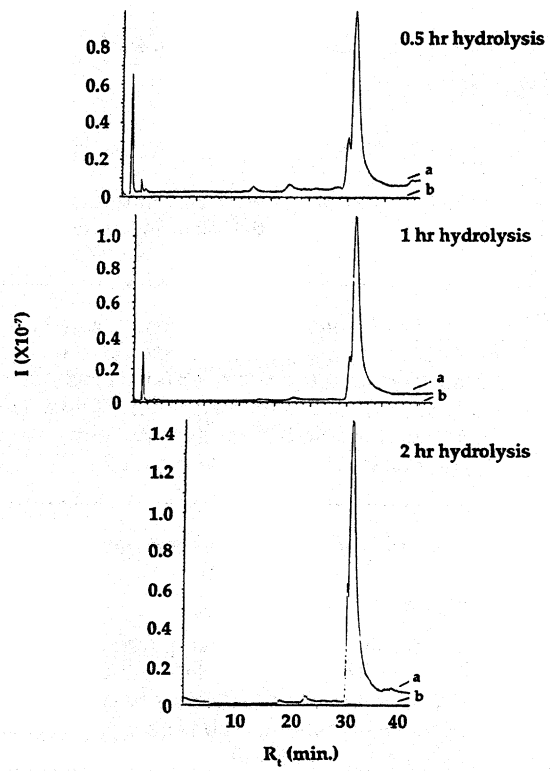

Figure 2. HPLC–MS of enzymatically treated tomato cutin: (a) M_r 100–1500; (b) M_r 500–1500. The 2 and 24 h hydrolysis results were identical.

cutins because its monomer composition is relatively simple in comparison to that of other cutins. It is composed primarily of 10,16- (77%) and minor amounts of 8,16- (9%) and 9,16- (12%) dihydroxyhexadecanoic acids (Holloway, 1982). An HPLC–chemical ionization mass spectrometry (CIMS) method was developed for the analysis of cutin hydrolysis products which includes the detection of oligomers as well as monomeric products. Since tomato cutin monomers and the corresponding ester derivatives have molecular weights (M_r) between 300 and 400, the presence of molecular or pseudomolecular ions above m/z 500 in the CIMS are indicative of cutin oligomers. The application of this technique to establish optimum hydrolysis conditions for oligomer production can be used to demonstrate the method.

Both chemical and enzymatic hydrolysis conditions were examined to determine optimum conditions for yielding cutin oligomers. Hydrolysis of tomato cutin in 1.5 M methanolic KOH was examined over a 4 h time period. The HPLC-CIMS of the 0.5 h hydrolysis products is shown in Figure 1. The monomer composition based on relative retention time (RR_t) and M_r (Table 1) is consistent with previous results (Gerard et al., 1992b). Monomer concentration increased throughout the time course of the experiment; however, dimer concentration maximized within the first hour (Table 2). In contrast to chemical hydrolysis, no compounds in the M_r range 500–1500 were observed during enzymatic hydrolysis (Figure 2), which suggests that the enzyme, in this case *F. solani* cutinase, is an exoenzyme (the mechanism of cutinase activity is being examined further). On the basis of these results, chemical hydrolysis appears to be the best method for obtaining oligomeric fragments.

The structures of oligomers obtained by the partial hydrolysis of tomato cutin were tentatively characterized by correlating the molecular weight of the oligomer

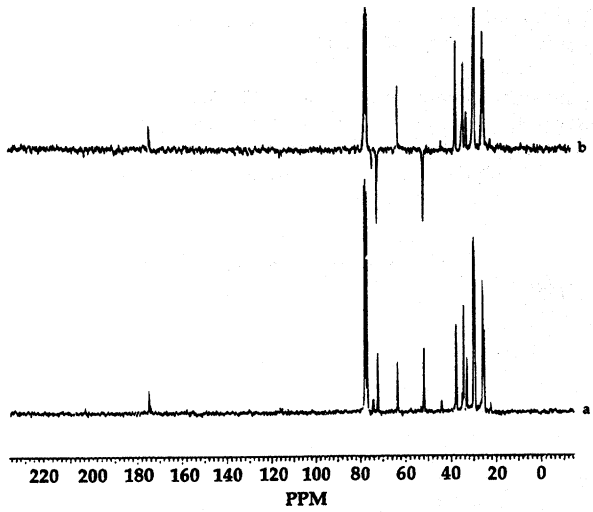

Figure 3. (a) ^{13}C NMR and (b) APT NMR of purified dimer from partially hydrolyzed tomato cutin.

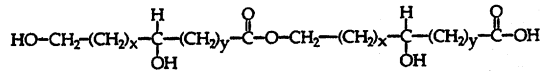

Figure 4. Structure of the dimer isolated from the partial hydrolysate of tomato cutin.

with the monomer composition, i.e., dimer M_r = [monomer$_x$ M_r + monomer$_y$ M_r] − 18. To test the validity of these assignments, we isolated the compound eluting at 35 min (Figure 1). This peak was chosen because, as can be seen in a comparison of the total ion chromatogram (peaks 1−11) with ions in the m/z 500−1500 range (peaks a−f), there are no monomers, i.e., compounds of M_r < 500, in this peak. The peak was isolated in milligram quantities by column and thin-layer chromatography and shown to be predominantly one molecular weight species, M_r = 572. Acetylation of the purified compound yielded a compound of M_r = 698, indicating the presence of three hydroxyls. Methanolic KOH hydrolysis or BF_3/MeOH treatment yielded a mixture of dihydroxyhexadecanoic acid methyl esters that consisted primarily of methyl 10,16-dihydroxyhexadecanoate.

The APT NMR data (Figure 3), which give positive signals for HCO and negative for H_2CO, support the proposed structure of the dimer in which the ester link involves the ω-hydroxyl exclusively (Figure 4) since a carbon resonance for only one HCOR group is apparent (63.4 ppm). If the ester linkage was formed by reaction with the C10 hydroxyl, two positive resonances should be observed in this region of the APT spectrum (R = OCR′ or H), whereas these groups are equivalent in the linear dimer (R = H). The minor oligomers a−d (Figure 1) have been tentatively identified as isomers of the dihydroxyhexadecanoic acid dimer. It remains to be determined whether any of these isomers are branched and/or linear.

The introduction of the cutin hydrolysates via the particle-beam separator has its limitations. Volatility is a factor that probably accounts for not observing oligomers higher than dimers (diolein has a 50 times greater response factor than triolein under the same conditions). We are now optimizing MS conditions and preparing more volatile derivatives, e.g., TMSi and acetate derivatives, in an attempt to observe these higher molecular weight oligomers. Another approach would be to use a technique such as secondary ion mass spectrometry; however, the complexity of the hydrolysate requires some form of chromatography prior to mass spectral analysis. We have explored the use of HPLC gel filtration fractionation prior to analysis as a possible purification technique; however, fractionation of a synthetic cutin-like oligomer mixture prepared in this laboratory (Osman et al., 1993) gave poor resolution under the conditions described under Experimental Procedures.

One of the objectives of our research program is to try to identify hydrolysis fragments that would be indicative of cross-link sites. These fragments should be observed by our analytical method unless they are too low in volatility. Our analyses, to date, with tomato cutin have revealed no such structures. Previous studies on the structure of cutin (Chamel and Marechal, 1992; Ramirez et al., 1992) have indicated that the polymer is highly cross-linked. These results are based on reaction of the polymer prior to hydrolysis; incomplete reaction would give an erroneously high number of cross-links. As our method now stands, cross-linked sites, because of their high molecular weight (i.e., containing two hydroxyfatty acids) and the cross-linking substituent, may elude analysis. However, monomers with cross-linkable substituents should be possible to detect. Increasing the volatility of the oligomers should make analysis of cross-linked sites feasible.

To our knowledge, this is the first report of the characterization of a cutin oligomer. Stark et al. (personal communication) have isolated a high molecular weight material from lime cutin which has yet to be completely characterized. Since it has now been demonstrated that it is possible to obtain dimers by partial chemical hydrolysis, it is very likely that higher molecular weight species are present in the same hydrolysates, which upon characterization should result in a greater understanding of the cutin polymer structure.

LITERATURE CITED

Chamel, A.; Marechal, Y. Characterization of isolated plant cuticles using Fourier transformed infrared (FTIR) spectroscopy. *C. R. Acad. Sci. Paris, Ser. 3* **1992**, *315*, 347−354.

Deas, A. B. H.; Holloway, P. J. The intermolecular structure of some plant cutins. In *Lipids and Lipid Polymers in Higher Plants*; Tevini, M., Lichtenthaler, H. K., Eds.; Springer-Verlag: Berlin, 1977; pp 293−299.

Fett, W. F.; Gerard, H. C.; Moreau, R. A.; Osman, S. F.; Jones, L. E. Cutinase production by *Streptomyces* spp. *Curr. Microbiol.* **1992a**, *25*, 165−171.

Fett, W. F.; Gerard, H. C.; Moreau, R. A.; Osman, S. F.; Jones, L. E. Screening of nonfilamentous bacteria for the production of cutin degrading enzymes. *Appl. Environ. Microbiol.* **1992b**, *58*, 2123−2130.

Garbow, J. R.; Ferrantello, L. M.; Stark, R. E. ^{13}C nuclear magnetic resonance study of suberized potato cell wall. *Plant Physiol.* **1989**, *90*, 783−787.

Gerard, H. C.; Moreau, R. A.; Fett, W. F.; Osman, S. F. Separation and quantitation of hydroxy and epoxy fatty acids by high-performance liquid chromatography with an evaporative light-scattering detector. *J. Am. Oil Chem. Soc.* **1992a**, *69*, 301−304.

Gerard, H. C.; Osman, S. F.; Fett, W. F.; Moreau, R. A. Separation, identification and quantitation of monomers from cutin polymers by high-performance liquid chromatography and evaporative light-scattering detection. *Phytochem. Anal.* **1992b**, *3*, 139−144.

Holloway, P. J. The chemical constituents of plant cutins. In *The Plant Cuticle*; Cutler, D. F., Alvin, K. L., Price, C. E., Eds.; Academic Press: New York, 1982; pp 45−85.

Kolattukudy, P. E. Biosynthesis and degradation of lipid polymers. In *Advances in Phytochemistry*; Loewus, F. A., Runeckles, V. C., Eds.; Plenum Press: London, 1977; Vol. 11, pp 185–246.

Kolattukudy, P. E. Cutin, suberin and waxes. In *Biochemistry of Plants*; Stumpf, P. K., Ed.; Academic Press: New York, 1980; Vol. 4, pp 571–643.

Osman, S. F.; Gerard, H. C.; Moreau, R. A.; Fett, W. F. Model substrates for cutinases. *Chem. Phys. Lipids* **1993**, *66*, 215–218.

Ramirez, F. J.; Luque, P.; Heredia, A.; Bukovac, M. J. Fourier transform IR study of enzymatically isolated tomato fruit cuticular membrane. *Biopolymers* **1992**, *32*, 1425–1429.

Zlotnik-Mazori, T.; Stark, R. E. Nuclear magnetic resonance studies of cutin, an insoluble plant polyester. *Macromolecules* **1988**, *21*, 2412–2417.

Received for review January 24, 1995. Accepted June 5, 1995.[®] Reference to brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.